TECHNICAL MANUSCRIPT 158

RELATIONSHIP BETWEEN COMPOUND STRUCTURE AND INFECTIVITY EFFECT ON EEE VIRUS

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RELATIONSHIP BETWEEN COMPOUND STRUCTURE AND INFECTIVITY EFFECT ON EEE VIRUS

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ABSTRACT

A number of sulfhydryl (-SH) compounds and the nonsulfur analogs of some of these compounds were tested for their effect on the infectivity of partially purified eastern equine encephalitis virus during storage at 4°C. Thiourea was found to be the most effective stabilizer, whereas urea and guanidine caused a loss of viral infectivity. Thiosemicarbazide and semicarbazide showed effects similar to those of thiourea and urea. Viral inactivation produced by p-chloromercuribenzoate, sodium thioglycollate, or sodium ascorbate could be prevented by thiourea. No correlation was found between the oxidation-reduction potential of virus suspensions containing the various compounds and the infectivity titer, or change in titer, of these suspensions. The presence of an -SH group in the molecule is not a requisite for stabilization: glutamine and dipyridyl exhibited good stabilizing properties.

I. INTRODUCTION

The stabilizing effect of cysteine on eastern equine encephalitis (EEE) virus was demonstrated by Bang and Herriott[1] and later by Labzoffsky.[2,3] These authors suggested that the reducing power of cysteine suppressed inactivation of the virus by inhibiting oxidation. However, other reducing agents tested by Labzoffsky had no stabilizing effect. In 1958, Pohjanpelto[4] showed that the thermal stability of a heat-sensitive strain of poliovirus was increased by incubation of the virus with L-cystine. Recently, Wallis and Melnick[5] reported that thermoresistant strains of poliovirus were developed by passage of the virus in tissue culture systems containing cystine, and that the reduction of disulfide bonds in these strains caused a loss of thermostability. In 1963, Philipson[6] reviewed information dealing with the reactions of the sulfhydryl groups of enteroviruses that suggested that sulfhydryl groups play a definite role in the early stages of the interaction of enteroviruses with host cells. Philipson and Choppin[7] reported that EEE virus resembled the enteroviruses and differed from the other arboviruses tested in that its hemagglutinating activity was inactivated by the sulfhydryl group reagent, p-chloromercuribenzoate.

We have tested a number of compounds, including some sulfhydryl compounds and their nonsulfur analogs, for their effect on the infectivity of partially purified EEE virus during storage at 4°C.

II. METHODS

EEE virus used in these tests was partially purified from Maitland type chick embryo cultures by one cycle of differential centrifugation, followed by two rinses of the viral sediment with phosphate buffer. The viral sediment was then suspended in 0.02 M phosphate buffer of pH 7.8. Equal volumes of this virus suspension and of the compound being tested were mixed and stored at 4°C in filled, rubber-stoppered glass bottles. Control samples were prepared by mixing equal volumes of the virus suspension and phosphate buffer.

Infectivity was determined by titration in 14-day embryonated eggs inoculated via the amniotic cavity.

III. RESULTS

A. COMPARISON OF STABILIZING EFFECTS OF THIOUREA AND CYSTEINE

When thiourea and L-cysteine were compared for their relative stabilizing effect on EEE virus, thiourea was found to be effective at a tenfold lower concentration than cysteine, as shown in Table I. Samples were assayed for infectivity at intervals up to 14 days. Data from a number of other tests indicate that 0.01 M thiourea is as effective as 0.1 M cysteine over relatively long periods of storage (seven to ten weeks).

TABLE I. COMPARISON OF THIOUREA AND CYSTEINE AS STABILIZERS OF EASTERN EQUINE ENCEPHALITIS VIRUS

Sample	Infectivity for Embryonated Egg[a/] Time, days at 4°C					
	0	0.17	1	3	7	14
Control	8.2	8.2	7.0	4.8	4.1	3.8
Cysteine 0.1 M		8.1	6.9	7.4	7.0	6.8
0.01 M		7.6	7.1	6.5	4.8	3.4
0.001 M		6.4	4.5	<3.0	<3.0	-
Thiourea 0.1 M		7.5	7.2	6.9	7.4	6.9
0.01 M		7.3	8.1	6.8	7.6	7.4
0.001 M		7.1	7.0	6.6	5.9	5.2

a. $\log_{10}$ amniotic LD_{50} per ml.

B. COMPARISON OF EFFECTS OF SULFHYDRYL COMPOUNDS AND THEIR NONSULFUR ANALOGS

In a study of the aerosol stabilization of Serratia marcescens by metal-binding compounds, Zimmerman[8] demonstrated that a number of sulfhydryl compounds were excellent stabilizers, whereas their nonsulfur analogs were ineffective. Because thiourea was found to stabilize EEE virus, we were interested in determining the effect of the structurally related compounds, urea and guanidine, on this virus. The effect of these compounds at the

0.01 M level is shown in Figure 1. Urea and guanidine did not stabilize the virus, but instead increased the loss of viral infectivity. Note that in the presence of thiourea the infectivity decreased only 0.5 log in 25 days.

Semicarbazide and thiosemicarbazide were also compared for their effect on EEE virus. (Figure 2). Samples containing 0.01 M semicarbazide showed greatly decreased titers at the first interval tested (24 hours); in contrast, thiosemicarbazide stabilized the virus.

C. PREVENTION OF SPECIFIC INACTIVATION OF EEE VIRUS BY THIOUREA

It was found that the reducing agents, sodium thioglycollate and sodium ascorbate, and the sulfhydryl group reagent, p-chloromercuribenzoate, caused inactivation of partially purified EEE virus. Inactivation by these compounds did not occur when thiourea was present. In contrast to the relatively slow inactivation of EEE virus produced by thioglycollate and p-chloromercuribenzoate, sodium ascorbate caused very rapid inactivation of the partially purified virus, as indicated in Figure 3. Inactivation curves for 10^{-3} M and 10^{-4} M ascorbate at pH 7.8 are shown. 10^{-3} M ascorbate caused a loss of infectivity of more than 3.0 log in 2½ minutes. Inactivation by ascorbate could be prevented by the addition of equimolar amounts of thiourea either before the addition of ascorbate or together with ascorbate.

D. COMPARISON OF THE INACTIVATING EFFECTS OF SEVERAL COMPOUNDS

Table II shows the extent of inactivation produced by compounds that were found to be inactivating to EEE virus. Both p-chloromercuribenzoate and iodoacetamide at alkaline pH levels are known to react with sulfhydryl groups in proteins, but these reagents also react with other protein sites. None of the compounds caused the sharp, rapid inactivation that was produced by ascorbate. Inactivation of poliovirus by ascorbic acid was reported in 1935 by Jungeblut.[9] In a 1953 review,[10] the bacteriocidal effect of ascorbic acid was attributed to some product of the auto-oxidation of ascorbic acid, such as dehydroascorbic acid or hydrogen peroxide. However, Ericsson and Lundbeck,[11] in a 1955 study that included polio and influenza viruses, concluded that formation of hydrogen peroxide cannot be the essential mechanism of this antimicrobial effect. Our experiments indicate that dehydroascorbic acid but not hydrogen peroxide could cause the inactivation of EEE virus and that cysteine as well as thiourea will prevent this inactivation.

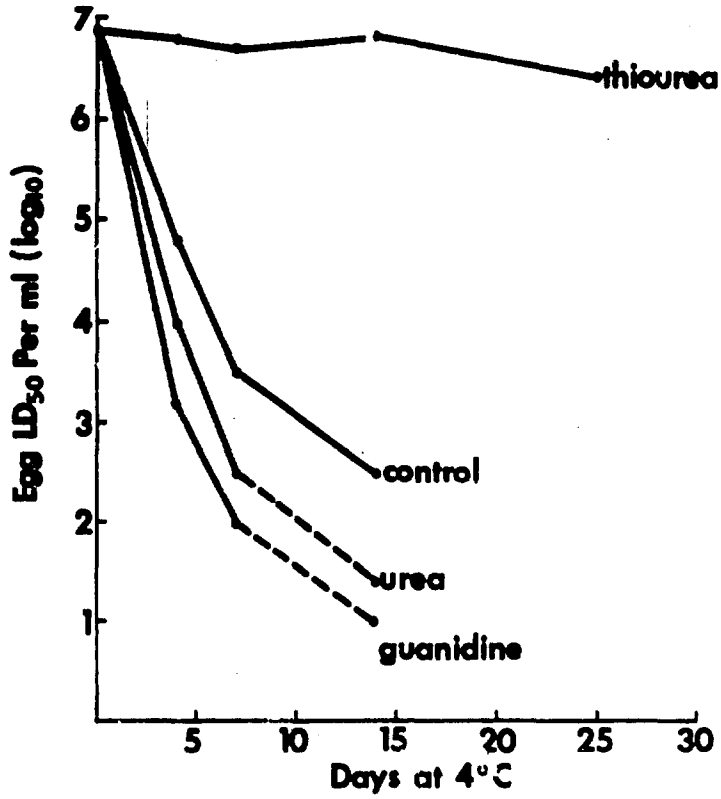

Figure 1. Comparisons of the Effects of Thiourea, Urea, and Guanidine at Concentrations of 0.01 M on the Infectivity of EEE Virus.

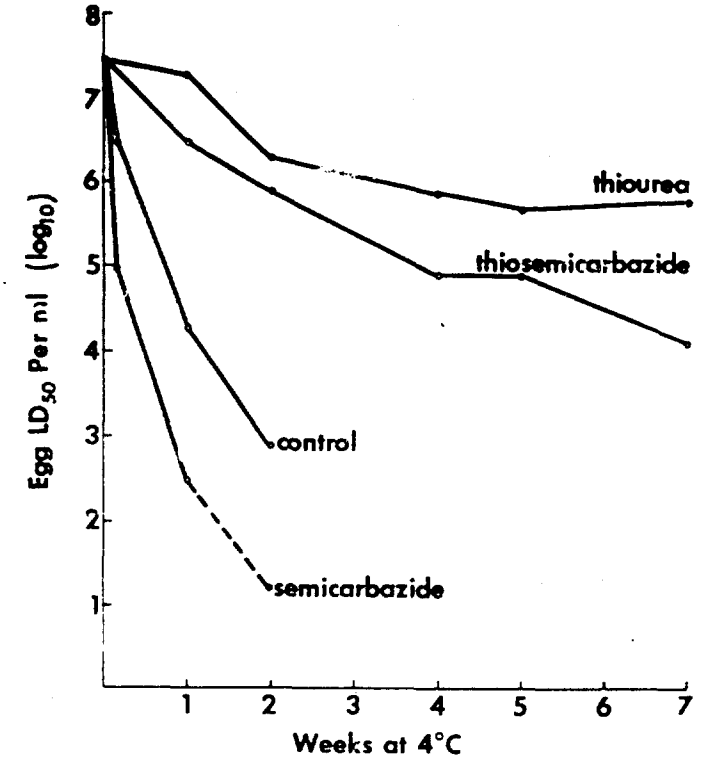

Figure 2. Comparisons of the Effects of Semicarbazide and Thiosemicarbazide at Concentrations of 0.01 M on the Infectivity of EEE Virus.

TABLE II. COMPARISON OF THE INACTIVATING EFFECT OF SEVERAL COMPOUNDS ON THE INFECTIVITY OF PARTIALLY PURIFIED EEE VIRUS AT 4°C

Compound	Concentration, M	Extent of Inactivation
Ascorbate	1×10^{-3}	5.0 log in 5 min
Ascorbate	1×10^{-4}	4.5 log in 2 hr
Thioglycollate	1×10^{-4}	2.5 log in 24 hr
p-Chloromercuribenzoate	1×10^{-4}	2.0 log in 24 hr
Iodoacetamide, pH 9	1×10^{-2}	1.5 log in 48 hr
Semicarbazide	1×10^{-2}	2.5 log in 24 hr
Urea or Guanidine[a]	1×10^{-2}	1 to 2 log in 72 hr

a. Inactivation with these compounds occurred under specific conditions of pH and virus purity.

E. COMPOUNDS HAVING STABILIZING OR INACTIVATING EFFECTS ON EEE VIRUS

Table III lists those compounds tested that showed stabilizing or inactivating effects on partially purified EEE virus. They are not arranged in order of increasing or decreasing activity. Cystine, when tested within the limits of its solubility, was as effective as cysteine at the same concentration levels; it is included parenthetically with cysteine. The sulfur-containing compounds, ethylene thiourea, glutathione and methionine were not as effective as thiourea and did not give consistent stabilization from experiment to experiment. The two compounds, glutamine and dipyridyl, which do not contain sulfur, exhibited, in some experiments, stabilizing properties that approached those of thiourea. Notice that one sulfhydryl compound, sodium thioglycollate, was found to be inactivating to EEE virus.

Because of the reducing nature of a number of the compounds tested we attempted to determine if any correlation existed between the oxidation-reduction potential of virus suspensions containing the various compounds and the infectivity titer, or change in titer, of these suspensions. No correlation has been found. For example, samples of virus that contained 0.01 M urea, guanidine, or thiourea all had approximately the same

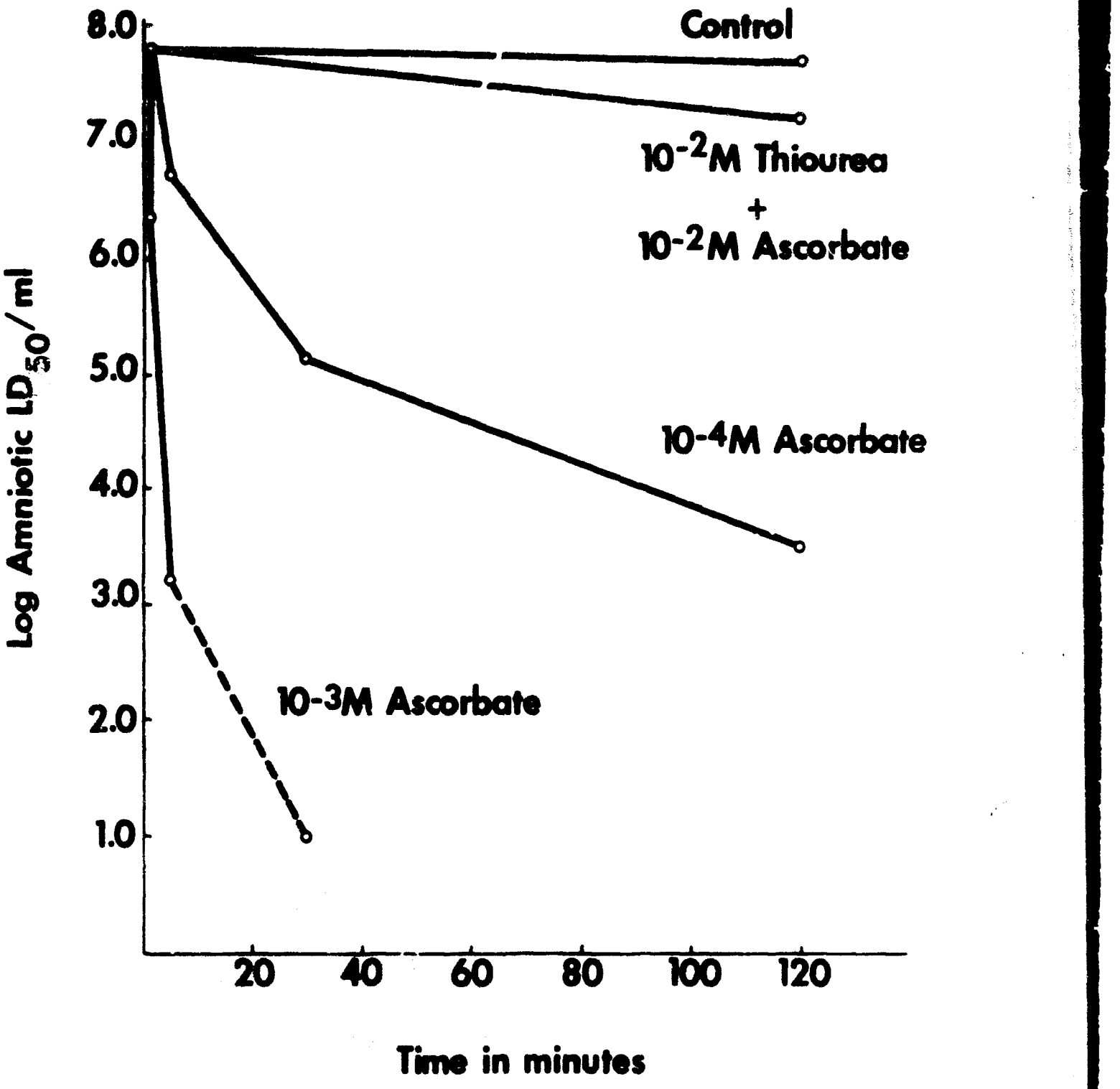

Figure 3. Inactivations of Partially Purified EEE Virus by Sodium Ascorbate.

reduction potential, yet the samples containing urea and guanidine had infectivity titers 3.0 log lower than the samples containing thiourea. We have found (as indicated in Table III) that some reducing agents are good stabilizers and others are highly inactivating.

TABLE III. COMPOUNDS WITH STABILIZING OR INACTIVATING EFFECTS ON EEE VIRUS

Stabilizing	Inactivating
Thiourea	Urea
Cysteine (Cystine)	Guanidine
Ethylene thiourea	Ascorbate
Thiosemicarbazide	Semicarbazide
Glutathione	Thioglycollate
Methionine	p-Chloromercuribenzoate
Glutamine	Iodoacetamide
Dipyridyl	

IV. DISCUSSION

Although the data obtained in this study suggest some relationship between the -SH group of an additive and the stabilization of viral infectivity, it has also been shown that compounds that contain no sulfur can act as stabilizers. However, it may well be that -SH groups of the viral protein are involved with both sulfur and nonsulfur additives in mechanisms of stabilization and inactivation. A possible mechanism for stabilization could be based on the postulation of Pohjanpelto,[4] who suggested that cystine stabilized poliovirus by combining with -SH groups of the viral protein, with subsequent cross linking between protein molecules thus preventing oxidation associated with an uncoiling of protein chains. An extension of this idea might be that through favorable reversible complexing,

-SH groups are protected and made available for eventual interaction with the host cell. Inactivation of viral infectivity by an added compound could result from (a) immobilization or alteration of -SH groups on the viral surface through irreversible complexing or other interaction with the compound, or (b) by the compound reacting with surface groupings in such a way as to cause a break in cross links with a resultant unfolding of protein chains to render the virus more susceptible to oxidative inactivation.

If the ultimate cause of loss of viral infectivity is disruption of the integral structure of viral nucleic acid by products (possibly free radicals) of some process (such as oxidation), then we might postulate that compounds stabilize viral infectivity by preventing this process from occurring either through complexing with traces of catalytic metal ions, buffering against unfavorable oxidation-reduction change, or reacting with sites on the viral protein so as to produce a more closely-knit protective coat for the sensitive nucleic acid. In a parallel sense, compounds might be inactivating to viral infectivity by in themselves initiating the inactivating process, by helping to establish conditions favorable to an acceleration of this process, or by causing an unfolding of the viral protein, thus making the nucleic acid more accessible to the inactivating mechanism. In another sense, compounds could be inactivating by altering the surface structure of the virus particle so as to prevent the interaction of virus and host cell.

V. SUMMARY

These experiments have established no single essential mechanism or factor as being responsible for the stabilization or inactivation of EEE virus. The data, however, indicate (a) that structurally-related compounds can exert entirely different effects on the infectivity of the virus, (b) that apparent alteration of sulfhydryl groups of the protein of EEE virus directly affects the viral infectivity or the stability of the virus, (c) the presence of a reducing agent or the establishment of a certain reduction potential does not, in itself, guarantee or impart stability to a suspension of EEE virus.

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